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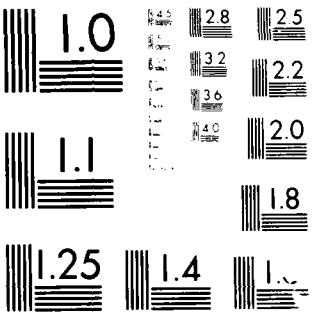

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IMMUNOLOGIC AND GENETIC SELECTION OF ADENOVIRUS VACCINE STRAINS: Synthesis and Characterization of Adenovirus Antigens

Final Report

by

Harold S. Ginsberg, M.D.

August 1984
(For the period August 1960-January 1982)

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Type 7 adenovirus
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L1 region
IIIa protein region
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Late proteins (CONTINUED)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

H7ts88 replicates in 293 cells at 39.5^{0}C although it physically maps in the L1 region of the genome, which encodes the IIIa protein. However, it expresses E1a and E1b gene products normally. At 39.5^{0}C it produces large amounts of the hexon protein, which is responsible for neuralizing antibodies, as well as all other functional late proteins except IIIa, but it does not produce infectious virus at the non-permissive temperature.
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The penton base (protein III) and the fiber (protein II) were characterized, which had not been previously done. The penton base had a molecular weight of 68,000 and the fiber had a molecular weight of 30,000 to 33,000. Previously, the fiber had been identified incorrectly.

Fifty conditionally lethal, temperature-sensitive (ts) mutants of the type 7 adenovirus vaccine strain have been isolated and characterized. Genetic analyses indicate that forty-nine of the mutants could be divided into 10 complementation groups. Selected mutants from each group were localized on the adenovirus genome by recombination and marker rescue analyses. The mutations were predominantly located in the late transcription regions L1, L2, and L3 which code for the 55-58K and IIIa proteins (L1), the penton base and pV1 (L2), and hexon protein (L3). It was striking that only one mutation involved an early gene product, the DNA-binding protein. The IIIa protein mutants are potentially excellent candidates for use in an attenuated, live virus vaccine since it produces large amounts of hexons which is the primary antigen responsible for neutralizing antibodies. Indeed, these mutants produce as much immunologically reactive hexons, fiber, and penton base as does wild-type 7 adenovirus. This contract period was devoted to characterization of these mutants in greater detail, particularly H7ts88.

19., con't.

Penton base protein
Fiber protein
293 cells
Type 4 adenovirus
Type 5 adenovirus
Soluble viral antigens
Virus-infected cells
Structural proteins
Virion morphology
Neutralizing antibodies
Viral uncoating
Capsid proteins
Hexon crystals
Synthesis capsid proteins
Hexon polypeptides
Hexon neutralizing antibodies
Hexon transport
100K nonstructural protein

FINAL REPORT DADA

SUMMARY

The research supported during the tenure of this contract has produced data that described the major components of the adenovirus capsid. These capsid proteins were purified, their immunological properties were defined, and the hexon was identified as the predominant antigen that induces neutralizing antibodies. The hexon was crystalized, its polypeptide structure characterized, and the antibodies directed against the hexon polypeptides were shown not to react with native hexons. Conditionally lethal, temperature-sensitive mutants of type 5 adenovirus were isolated, characterized, and studied to reveal essential features of formation of the capsomers and the assembly of the virions. Temperature-sensitive mutants of type 7 adenovirus were isolated from the accepted Armed Forces vaccine strain, and they were characterized to identify a mutant to serve as a better prototype vaccine strain. A mutant in the IIIa protein gene was considered the best ts Ad7 virus as a candidate vaccine strain since infection with it produced normal amounts of hexons without formation of infectious virus at the non-permissive temperature. Final characterization of the ideal virus for use in a vaccine was interrupted by cancellation of this contract.

INTRODUCTION

At the time that this contract was initiated there was only superficial and limited information available characterizing the adenovirus infectious particles and the biochemical reactions required for viral replication. These data, however, were deemed essential to approach prophylaxis and possible antiviral treatment of the numerous infections for which adenoviruses were considered to be etiologic agents. Initially, the studies to be summarized were directed towards a detailed characterization of the adenovirus structural proteins and identification of the viral protein(s) responsible for inducing neutralizing antibodies. Attention was next turned toward obtaining a better, and possibly safer, attenuated virus vaccine since type 7 adenovirus was known to be oncogenic in rodents. The Armed Forces' type 7 vaccine strain was used to derive and characterize conditionally lethal, temperature-sensitive mutants.

1.* Purification and immunological characterization of types 4 and 5 adenovirus soluble antigens.

Summary. A chromatographic method for the separation and isolation of adenovirus-induced antigens from homogenates of infected cells is described. This procedure, in conjunction with immunological techniques, has been employed to identify, characterize, and compare the soluble antigens of types 4 and 5 adenovirus-infected HeLa cells. By such means it was shown that adenovirus-infected cells of various origins produce three soluble antigens in addition to infectious virus. These include a highly type-specific complement-fixing antigen, a group-specific or common complement-fixing antigen, and a toxin-like material which, when added to normal cells, causes rapid degenerative changes. The immunological behavior of the toxin is that of a group-specific or common adenovirus antigen. It was further shown that similar antigens from types 4 and 5 adenovirus-infected HeLa cells exhibited strikingly different chromatographic characteristics.

2. Effect of proflavine on the synthesis of adenovirus, type 5, and associated soluble antigens.

The synthesis of type 5 adenovirus in HeLa cells was suppressed to a considerable extent by low concentrations of proflavine, an acridine dye. In comparison, the processes leading to the production of soluble complement-fixing antigens and toxin were less sensitive to the action of this chemical. Addition of proflavine to infected cells at different times during the virus growth cycle revealed that the processes leading to the synthesis of soluble antigens began prior to the first appearance of newly synthesized virus. This observation is compatible with the hypothesis that the soluble antigens may represent virus subunits or precursor materials. In addition, these data indicate that it is possible to interrupt the latter stages of the virus synthetic process by addition of proflavine late in the eclipse period.

* Refers to number of publications listed at end of report.

3. Protein synthesis in type 5 adenovirus-infected cells. Effect of p-fluorophenylalanine on synthesis of protein, nucleic acids, and infectious virus.

Synthesis of specific proteins in adenovirus-infected HeLa cells was studied utilizing the amino acid analog p-flourophenylalanine. Virus multiplication and the production of specific soluble antigens could be completely suppressed by addition of this compound. Two distinct stages were revealed within the virus eclipse period when the presence of analog prevented virus synthesis. The first or early effect of p-flourophenylalanine appeared to result from interference with production of functional enzymes necessary for synthesis of virus structural protein. An overall picture of the temporal relationships between DNA, protein, and virus synthesis was obtained by conducting inhibition studies in parallel employing 5-flourodeoxyuridine, an inhibitor of thymidilate synthetase, as well as p-flourophenylalanine. These studies revealed that virus DNA synthesis preceded the production of virus-specific protein by approximately 2 hours and the synthesis of mature infectious virus by about 4 hours.

4. Structure of type 5 adenovirus. I. Antigenic relationship of virus-structural proteins to virus-specific soluble antigens from infected cells.

Type 5 adenovirus was purified by fluorocarbon (freon 113) treatment followed by banding in a CsCl equilibrium density gradient. This method permitted separation of virus from normal host cell materials and virus-specific soluble antigens. Virus banded in CsCl with a mean bouyant density of 1.3349 gm./cm³. The three virus-specific soluble antigens (group- and type-specific antigens and toxin) banded together with a mean bouyant density of 1.2832 gm./cm³. The group-specific antigen was the predominant antigen of the purified virus particle, whereas the group- and type-specific antigens were present in equal titers in the antigen band. Infectious virus particles were inactivated by prolonged dialysis at pH 10.5. Centrifugation of inactivated virus preparations in a CsCl equilibrium density gradient resulted in separation of virus DNA from specific antigen: the antigens banded with a mean bouyant density of 1.2832 gm./cm³ and the DNA sedimented to the bottom of the tube. The predominant antigen derived from purified virus particles was the group-specific antigen and it was in the same relative proportion to the type-specific antigen as measured in intact particles. The antigens derived from disrupted virus were immunologically identical with the soluble virus antigens present in infected cells.

5. Structure of type 5 adenovirus. II. Fine structure of virus subunits. Morphologic relationships of structural subunits to virus-specific soluble antigens from infected cells.

Purified type 5 adenovirus was disrupted at pH 10.5 and the capsid shown to be comprised of two characteristic morphological subunits: (*a*) Hollow, polygonal structures corresponding to the virus capsomeres seen in preparation of purified virus and (*b*) thread-like strands also identifiable in preparations of purified virus. These structures were compared morphologically with purified preparations of the group- and type-specific soluble antigens characteristically produced in mammalian cells infected with adenoviruses. The group-specific soluble antigen was a homogeneous preparation of hollow, polygonal rods identical with the virus capsomeres. The type-specific soluble antigen corresponded to the thread- or fiber-like components of the purified virus particle. Inspection of disrupted virus preparations confirmed earlier immunological data which indicated that the major virus antigen was the group-specific soluble antigen. These data provide convincing evidence for the hypothesis that the adenovirus-induced soluble antigens represent virus subunits produced in excess.

6. Production of specific neutralizing antibody with soluble antigens of type 5 adenovirus.

Injection into rabbits of virus-free soluble antigen preparations obtained from type 5 adenovirus-infected HeLa cells results in production of specific virus neutralizing antibody. The neutralizing antibodies are present in antisera in high titer and are completely type-specific. This is interpreted as evidence that the virus-induced soluble antigens are structurally related to virus protein. Adsorption studies employing purified group-specific (L) antigens derived from types 2, 4, and 5 virus-infected cells and antiserum specific for the type 5 L antigen reveal basic immunological differences in these "common group" antigens. The type 5 L antigen cross-reacted with type 2 and 4 antigens, however, the type 5 antigen, in addition, had an antigenic reactivity which was type-specific.

7. Characterization of a new viral component of type 5 adenovirus by immunoelectrophoresis.

In extracts of adenovirus infected cells the presence of 3 viral soluble antigens has been reported. The soluble antigens represent structural subunits of the virus produced in excess, and they are identical to the antigens isolated from the virus particle itself.

In this report a new antigen of the type 5 adenovirus virion will be discussed. The antigen could be detected by immunoelectrophoresis of purified virus disrupted by dialysis against pH 10.5 bicarbonate buffer, and only if the antiserum used for the immunodiffusion was made against purified virus. The antigen could not be found in the soluble antigen fraction. The new antigen was separated from the 3 known viral antigens by immunoelectrophoresis, and it could be demonstrated that the new antigen is immunologic from the 3 other antigens.

The new viral component could be separated from 2 of the 3 other antigens by rate zonal centrifugation in a linear sucrosegradient.

8. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid.

Highly purified, ^{32}P labeled type 5 adenovirus was employed to study "uncoating" of viral deoxyribonucleic acid (DNA)—defined as the development of sensitivity to deoxyribonuclease. Viral infectivity and radioactivity adsorbed to KB cells at the same rate and significant amounts of ^{32}P did not elute from cells throughout the eclipse period. Kinetic studies of viral penetration, eclipse of infectivity, and uncoating of viral DNA indicated that the three events were closely related temporally, that the rates of each were similar, and that they were completed within 60 to 90 min after infection. Viral penetration, eclipse, and uncoating proceeded normally under conditions which blocked protein synthesis, but they did not occur at 0 to 4 C. Neither viral DNA nor viral protein was degraded to acid soluble material during the eclipse period. The nature of adenovirus DNA was studied after it was converted intracellularly from deoxyribonuclease-resistant to deoxyribonuclease-susceptible. Intact virions centrifuged in sucrose gradients had a sedimentation coefficient of approximately 800, and viral DNA sedimented as a particle of about 30S. Infection of KB cells with purified ^{32}P labeled virus yielded deoxyribonuclease-susceptible viral nucleic acid which was in particles with sedimentation coefficients of 350 to 450S, i.e., greater than 10 times faster than DNA obtained from purified virions which had been disrupted by exposure to *p*H 10.5. When the DNA from disrupted virions was mixed with cell lysates, its sedimentation characteristics were essentially unchanged by the presence of cellular material.

9. Mechanism by which fiber antigen inhibits multiplication of type 5 adenovirus.

Purified fiber antigen of type 5 adenovirus inhibited the multiplication of type 5 adenovirus by 50%, when 35 μg of fiber antigen protein was added to 10^6 KB cells in suspension culture. Although the fiber antigen reduced the number of virions adsorbed per cell when a multiplicity of infection of 50,000 plaque-forming units (PFU)/cell was employed, the number of cells infected was not diminished under these conditions. If a low multiplicity of infection (1.1 PFU/cell) was used, viral adsorption was not detectably decreased. The fiber antigen did not reduce the capability of virions to liberate their viral deoxyribonucleic acid (DNA). The biosyntheses of DNA, ribonucleic acid (RNA), and protein were blocked about 20 to 25 hr after the addition of fiber antigen to cultures of uninfected or type 5 adenovirus-infected KB cells. Most of the fiber antigen protein became cell-associated between 22 and 36 hr after it was added to cells. The hexon antigen neither inhibited viral multiplication nor blocked the biosynthesis of DNA, RNA, or protein. Moreover, the hexon did not attach to KB cells. The profound effects of the fiber antigen were not due to the induction of an interferon-like substance, for actinomycin D did not reduce the ability of the fiber to inhibit multiplication of type 1 poliovirus.

10. Inhibition of host protein synthesis in type 5 adenovirus-infected cells.

The effect of type 5 adenovirus infection on the synthesis of host-cell proteins by suspension cultures of KB cells was investigated. Although total protein synthesis continued at a constant rate for approximately 36 hr, net synthesis of five host enzymes (lactic dehydrogenase, acid phosphatase, deoxyribonuclease, fumarase, and phosphoglucose isomerase) was found to stop 16 to 20 hr after infection. The synthesis of alkaline phosphatase stopped 9 to 12 hr after infection. The inhibition of host protein synthesis occurred shortly after the synthesis of viral antigens had begun, accounting for the continued synthesis of total protein. An investigation of the relationship between synthesis of viral antigens and inhibition of host protein synthesis yielded results which suggest that the two processes are in some way coupled.

11. Role of adenovirus structural proteins in the cessation of host cell biosynthetic functions.

Two of the adenovirus capsid proteins, the fiber and the hexon, complexed with either KB cell or type 5 adenovirus deoxyribonucleic acid (DNA). Maximal binding occurred at 0.01 M NaCl; increasing the ionic strength of the reaction mixture to 0.2 M NaCl resulted in a decrease in the association of either antigen to DNA. Variations of *p*H between 6.3 and 8.4 did not affect the binding of fiber antigen to DNA. Below *p*H 7.5, however, there was a small decrease in the ability of the hexon to bind nucleic acid. The association between the adenovirus structural proteins and DNA was reversible and was independent of whether the DNA was native or denatured. The fiber or hexon protein inhibited the DNA-dependent ribonucleic acid (RNA) polymerase and the DNA polymerase from KB cells. On a weight basis, the fiber protein inhibited enzymatic activity to a greater extent than the hexon. Increasing the template DNA concentration decreased this inhibition. The inhibition of the DNA-dependent RNA polymerase activity by either antigen could be reversed by increasing the ionic strength of the reaction mixture. After infection of KB cells with type 5 adenovirus, the levels of DNA and RNA polymerases remained unchanged for 15 to 20 hr. Thereafter, the specific activity of both enzymes decreased. By 30 hr post-infection, the polymerase activities were only about 30% of the enzyme activities in uninfected cells.

12. Cytoplasmic synthesis of type 5 adenovirus capsid protein.

–That synthesis of viral capsid proteins is accomplished in the cytoplasm of cells infected with type 5 adenovirus was demonstrated with three independent methods: (1) autoradiography; (2) immunological coprecipitation of proteins on polyribosomes obtained by centrifugation of cytoplasmic extracts in linear sucrose gradients, and (3) hybridization between denatured viral DNA and RNA isolated from polyribosomes. Synthesis of the nascent viral polypeptide chains was accomplished rapidly on 200S polyribosomes, after which they were quickly released and transported into the nuclei.

13. Synthesis, transport, and morphogenesis of type 5 adenovirus capsid proteins.

During the period between 20 and 24 hr after infection of KB cells with type 5 adenovirus, at a time when approximately 85% of the proteins made were virus-specific, viral proteins were synthesized on polyribosomes with an average sedimentation coefficient of 200S. The polypeptide chains synthesized during a 1-min period of labeling with ^{14}C-amino acids had an average sedimentation coefficient of 3.4S in sucrose gradients containing 1% sodium dodecyl sulfate. Within 1 min after completion, the newly made polypeptide chains were released from polyribosomes, and the majority were transported into the nuclei within 6 min. Meanwhile, the immunological reactivity of the newly synthesized proteins also increased rapidly. During the same 6 min interval after synthesis, the single polypeptide chains assembled into multimeric proteins with average sedimentation coefficients of 6S, 9S, and 12S. The 6S and 12S proteins were identified immunologically as the fiber and hexon capsid proteins, respectively. The 9S protein was trypsin sensitive and appeared to be the precursor of the penton; it was tentatively identified as the penton base. The penton had a sedimentation coefficient of about 10.5S and sedimented with the hexon in sucrose gradients. The concomitant migration of nascent proteins into the nuclei, development of the capsid proteins' immunological reactivity, and morphogenesis of the multimeric capsid proteins suggest that the single polypeptide chains or small complexes were transported into the nuclei where they assembled into mature structural proteins of the virion.

14. Characterization of crystals of type 5 adenovirus hexon

Tetrahedral crystals of type 5 adenovirus hexon have been studied by X-ray diffraction. The crystals belong to the cubic space group $P2_13$ with $a = b = c = 149.9$ Å. The protein in the asymmetric unit probably represents one-third of a hexon. These crystals differ from the description of the tetrahedral crystals of type 5 adenovirus hexon reported by Macintyre, Pereira & Russell (1969) but are isomorphous with the bipyramidal-shaped crystals of type 2 adenovirus hexon studied by Franklin, Pettersson, Åkervall, Strandberg & Philipson (1971).

15. Synthesis in vitro of type 5 adenovirus capsid proteins

Reaction mixtures containing cytoplasmic extracts and ribosomal fractions prepared from KB cells infected with type 5 adenovirus were able to carry out incorporation of amino acids into protein. The in vitro product included proteins which reacted specifically with antisera to adenovirus capsid proteins; in control experiments with extracts from uninfected cells, no reactions with the antisera were found. The viral proteins were synthesized in vitro on small polyribosomes, were released from them, and significant numbers of the free polypeptides were assembled in vitro into multimeric adenovirus capsid structures.

16. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus

Eight temperature sensitive (ts) mutants that replicate normally at 32 C but poorly, if at all, at 39.5 C have been isolated from mutagenized stocks of a wild type strain of type 5 adenovirus. Three mutagens were employed: nitrous acid, hydroxylamine, and nitrosoguanidine. Ts mutants were isolated from mutagenized viral stocks with frequencies between 0.01 and 0.1%. All eight mutants had reversion frequencies of 10^{-8} or less. Complementation experiments in doubly infected cultures at the nonpermissive temperature separated the mutants into three nonoverlapping complementation groups. Complementation yields ranged from a 2.3- to a 3,000-fold increase over the sums of the yields from the two singly infected controls. Genetic recombination was also demonstrated; approximate recombination frequencies ranged from 0.1 to 15%. Preliminary biochemical and immunological characterization of the mutants indicated that (i) the single mutant in complementation group I did not replicate its deoxyribonucleic acid (DNA) or synthesize late proteins at the nonpermissive temperature but did inhibit host DNA synthesis to 25% of an uninfected control, (ii) the four group II mutants replicated viral DNA, shut off host DNA synthesis, synthesized penton base and fiber, but did not synthesize immunologically detectable hexon, the three mutants in complementation group III synthesized viral DNA, shut off host DNA synthesis, and made immunologically reactive capsid proteins (hexon, penton base, and fiber).

17. Hexon peptides of types 2, 3, and 5 adenoviruses and their relationship to hexon structure

Peptides of hexons from type 2 and 5 (subgroup III) and type 3 (subgroup I) adenoviruses were produced by treatment with cyanogen bromide and were separated by isoelectric focusing in polyacrylamide gels containing 8 M urea. Peptides with identical isoelectric points, but from different hexon types, were considered to have structural similarities. According to this criterion for chemical relatedness, about two-thirds of the type 2 and 5 hexon peptides may be considered similar. In contrast, the majority of the type 3 hexon peptides differed chemically from peptides of type 2 and 5 hexons. Virions and free hexons were iodinated with ^{125}I in the presence of lactoperoxidase and H_2O_2. When ^{125}I-labeled virions were disrupted and the hexon was purified, the highly labeled cyanogen bromide peptides had pI values greater than 6.8, some unique as well as some common peptides were labeled. When purified hexons from the excess cellular pool were iodinated, peptides common to types 2, 3, and 5 (peptides 12 and 14) were most extensively labeled. Thus, hexons assembled in virions and those free in solution were iodinated differently. The data suggest that immunologically the hexons in viral capsids react differently from unassembled hexons because the polypeptide chains assume slightly different folding configurations in the two hexon forms and therefore expose different regions of the protein to antibodies.

18. Antibody to the type adenovirus hexon polypeptide: Detection of nascent polypeptides in the cytoplasm of infected KB cells

Summary. Antibody to type 5 adenovirus hexon polypeptides reacted with the isolated hexon polypeptide chains (monomers) but not with the native multimeric hexon capsomers (trimers). Indirect immunofluorescence detected hexon polypeptides mainly in the cytoplasm of KB cells infected with types 2 and 5 adenoviruses. Cells infected with adenovirus mutants H5ts116 or H5ts115, which are defective in the production of immunologically detectable hexons, synthesized immunologically detectable hexon polypeptides at the nonpermissive temperature.

19. Polyamines in type 5 adenovirus-infected cells and virions

The incorporation of L-[^{14}C]ornithine and [^{14}C]putrescine into putrescine, spermidine, and spermine in type 5 adenovirus-infected KB cells was identical to that in uninfected control cells early in infection, but incorporation into putrescine stopped after 8 to 12 h and the rate of incorporation into spermidine was reduced between 12 and 20 h after infection. The amount of polyamines found associated with purified virus could neutralize a maximum of 3 to 4% of the virus DNA, but the small quantities of polyamines detected could not be distinguished from nonspecific binding of polyamines to virions. These data suggest that polyamines are probably not integral components of adenovirus particles.

20. Characterization of temperature-sensitive, hexon transport mutant of type 5 adenovirus

Infection of KB cells at 39.5°C with H5ts147, a temperature-sensitive (ts) mutant of type 5 adenovirus, resulted in the cytoplasmic accumulation of hexon antigen; all other virion proteins measured, however, were normally transported into the nucleus. Immunofluorescence techniques were used to study the intracellular location of viral proteins. Genetic studies revealed that H5ts147 was the single member of a nonoverlapping complementation group and occupied a unique locus on the adenovirus genetic map, distinct from mutants that failed to produce immunologically reactive hexons at 39.5°C ("hexon-minus" mutants). Sedimentation studies of extracts of H5ts147-infected cells cultured and labeled at 39.5°C revealed the production of 12S hexon capsomers (the native, trimeric structures), which were immunoprecipitable to the same extent as hexons synthesized in wild-type (WT)-infected cells. In contrast, only 3.4S polypeptide chains were found in extracts of cells infected with the class of mutants unable to produce immunologically reactive hexon protein at 39.5°C. Hexons synthesized in H5ts147-infected cells at 39.5°C were capable of being assembled into virions, to the same extent as hexons synthesized in WT-infected cells, when the temperature was shifted down to the permissive temperature, 32°C. Infectious virus production was initiated within 2 to 6 h after shift-down to 32°C; de novo protein synthesis was required to allow this increase in viral titer. If ts147-infected cells were shifted up to 39.5°C late in the viral multiplication cycle, viral production was arrested within 1 to 2 h. The kinetics of shutoff was similar to that of a WT-infected culture treated with cycloheximide at the time of shift-up. The P-VI nonvirion polypeptide, the precursor to virion protein VI, was unstable at 39.5°C, whereas the hexon polypeptide was not degraded during the chase. It appears that there is a structural requirement for the transport of hexons into the nucleus more stringent than the acquisition of immunological reactivity and folding into the 12S form.

21. Characterization of type 5 adenovirus fiber protein

Type 5 adenovirus fiber protein was purified and subjected to chemical characterization. Equilibrium sedimentation ultracentrifugation analysis indicated that the intact fiber has a molecular weight of approximately 183,000. Denaturation and chemical analyses implied that the fiber consists of three polypeptide chains, each of about 61,000 mol wt. Mapping of tryptic peptides and electrophoretic separation of the constituent chains suggested that the intact fiber consists of two identical and one unique polypeptide chains.

22. Purification and preliminary immunological characterization of the type 5 adenovirus, nonstructural 100K protein

The nonstructural 100,000-dalton (100K) protein of type 5 adenovirus was isolated and purified from infected KB cells by a combination of ion-exchange and affinity chromatographies. Rabbit antiserum containing specific 100K protein antibodies was used for indirect immunofluorescence examination of cells infected with wild-type virus, 100K mutants, and hexon mutants. The 100K protein, which is synthesized as a late protein, was observed primarily in the cytoplasm of cells infected with wild-type and mutant viruses.

23. Characterization of two temperature-sensitive mutants of type 5 adenovirus with mutations in the 100,000-dalton protein gene

Complementation analysis assigned the mutations of strains H5*ts*115 and H5*ts*116, two hexon-minus mutants, to the 100,000-dalton (100K) protein gene. Heterotypic marker rescue (i.e., type 5 adenovirus [Ad5] temperature-sensitive mutants DNA × *Eco*RI restriction fragments of Ad2 DNA) confirmed the results of previous marker rescue mapping studies, and the heterotypic recombinants yielded unique hybrid (Ad5-Ad2) 100K proteins which were intermediate in size between Ad5 and Ad2 proteins and appeared to be as functionally active as the wild-type 100K protein. Phenotypic characterization of these mutants showed that both the hexon polypeptides and the 100K polypeptides were unstable at the nonpermissive temperature, whereas fiber and penton were not degraded, and that the 100K protein made at 39.5°C could not be utilized after a shift to the permissive temperature (32°C). The role of the 100K protein in the assembly of the hexon trimer was also examined by in vitro protein synthesis. Normally, hexon polypeptides synthesized during an in vitro reaction are assembled into immunoreactive hexons. However, this assembly was inhibited by preincubation of the cell extract with anti-100K immunoglobulin G; neither anti-fiber immunoglobulin G nor normal rabbit immunoglobulin G inhibited hexon assembly. It is postulated that an interaction between the 100K protein and hexon polypeptides is required for effective assembly of hexon trimers.

24. Characterization of a temperature-sensitive fiber mutant of type 5 adenovirus and the effect of the mutations on virion assembly

A temperature-sensitive, fiber-minus mutant of type 5 adenovirus, H5*ts*142, was biochemically and genetically characterized. Genetic studies revealed that H5*ts*142 was a member of one of the three apparent fiber complementation groups which were detected owing to intracistronic complementation. Recombination analyses showed that it occupied a unique locus at the right end of the adenovirus genetic map. At the nonpermissive temperature, the mutant made stable polypeptides, but they were not glycosylated like wild-type fiber polypeptides. Sedimentation studies of extracts of H5*ts*142-infected cells cultured and labeled at 39.5°C indicated that a limited number of the fiber polypeptides made at the nonpermissive temperature could assemble into a form having a sedimentation value of 6S (i.e., similar to the trimeric wild-type fiber), but that this 6S structure was not immunologically reactive. When H5*ts*142-infected cells were shifted to the permissive temperature, 32°C, fiber polypeptides synthesized at 39.5°C were as capable of being assembled into virions as fibers synthesized in wild type-infected cells; de novo protein synthesis was not required to allow this virion assembly. In H5*ts*142-infected cells incubated at 39.5°C, viral proteins accumulated and aggregated into particles having physical characteristics of empty capsids. These particles did not contain DNA or its associated core proteins. However, when the infected culture was shifted to 32°C, DNA appeared to enter the empty particles and complete virions developed. The intermediate particles obtained had the morphology of adenoviruses, but they contained less than unit-length viral genomes as measured by their buoyant density in a CsCl density gradient and the size of their DNA as determined in both neutral and alkaline sucrose gradients. The reduced size of the intermediate particle DNA was demonstrated to be the result of incompletely packaged DNA molecules being fragmented during the preparative procedures. Hybridization of labeled DNA extracted from the intermediate particles to filters containing restriction fragments of the adenovirus genome indicated that the molecular left end of the viral genome preferentially entered these particles.

25. Selection and characterization of temperature-sensitive mutants of type 7 adenovirus

Fifty temperature-sensitive mutants of type 7 adenovirus have been isolated, following treatment of the vaccine strain of virus with hydroxylamine (36/398 plaques tested i.e., 9% mutation frequency) or nitrous acid (14/367 plaques tested i.e., 3.8% mutation frequency). The mutants were plaque purified 2 times on human foreskin (HF) cells, high titer stocks were grown and their growth characteristics were examined. The ratio of yields of these mutants at 39.5°C/32° ranged from 4.7x10-3 to 2.7x10-7, while their reversion frequencies (i.e., the ratio of plating efficiency at 39.5°C to that at 32°C) ranged from <2.7x10-4 to <2.2x10-7.

All but one of the 49 mutants examined made immunologically reactive late antigens at 39.5°C, as detectable by immunofluorescence. Forty eight of the mutants have been classified by complementation analyses. They were originally placed in 10 complementation groups (A-J) but a more detailed analysis revealed considerable overlap between groups B and C, and these are now thought to form a single group, whose members undergo intracistronic complementation. Similarly, H7ts55, the single member of group F, fails to complement two mutants of group G (H7ts62 and H7ts83), suggesting that it may well be a member of the latter group.

Intertypic complementation analyses were carried out between the Ad7 mutants and 6 Ad5 ts mutants in order to obtain preliminary data on the lesions induced in the Ad7 mutants. The genetic lesions of the Ad5 mutants used have been defined as follows:

H5ts128 - hexon gene
H5ts147 - hexon gene
H5ts116 -100K protein gene
H5ts142 - fiber gene
H5ts49 - IIIa protein gene
H5ts125 - DNA-binding protein gene

From the data obtained it is clear that 17 mutants, comprising groups B/C, D and E failed to complement both the hexon and the 100K mutants, suggesting that both gene products are necessary for assembly of functional hexons and that their interaction is type specific. Only one mutant (H7ts64, group D) is potentially a fiber mutant, since it gave a very low complementation index when crossed with H5ts142. The early mutant H7ts93, was the only mutant to fail to complement H5ts125. All mutants complemented H5ts49.

Genetic studies of temperature-sensitive mutants can give rise to some ambiguities. Such studies, therefore, were amplified by physical mapping techniques. The marker rescue technique was used to localize the sites of mutations along the genome for selected Ad7 mutants. The data obtained show that the mutations in members of complementation groups B/C and E are situated between map co-ordinates 50.4 and 60.2, which taken together with the genetic evidence, indicates that they are lesions in the hexon gene. Mutants in groups F/H and G were mapped between co-ordinates 34 and 36.7, which placed them in the L1 family of gene transcripts, coding for the 52/55K proteins and IIIa protein. Three of the mutants in complementation group J (H7ts21, H7ts38 and H7ts61) were mapped between co-ordinates 36.7 and 42, while the fourth member of the group, H7ts85, was located between co-ordinates 44 and 47. These data place members of group J in the L2 transcription unit, most probably in the penton base gene.

Phenotypic Characterization

Mutants in complementation groups B/C, D and E do not accumulate trimeric hexons in the nucleus at 39.5^{o}C, as detected by immunofluorescence, in agreement with genetic and physical mapping data. Mutants in complementation groups F/H, G, I and J accumulate similar amounts of hexon trimers in the nucleus at 39.5^{o}C as the WT virus.

Members of complementation groups F/H, G and I assemble empty capsids, which band at a bouyant density of 1.30gms/ml, as well as particles which band at a density of 1.26gms/ml, but fail to assemble DNA-containing virions. Analysis of the particles by electrophoresis in SDS-polyacrylamide gels, revealed that the empty capsids of the mutants did not contain proteins V and VII and that their pattern was indistinguishable from that of the empty capsids of WT virus. Particles with bouyant density of 1.26gms/ml consisted almost entirely of penton base and fiber, and were identical to those seen in WT lysates.

All the major viral structural proteins present in lysates of cells infected by the F/H and G group mutants at 39.5°C were immunologically reactive in an immunoprecipitation test, and polypeptides made by these viruses at the non-permissive temperature were stable upon prolonged chase at 39.5°C. Examination of DNA present in KB cells infected with H7ts88 (group G) at 39.5°C showed that the viral DNA was unit length and did not degrade upon chase. Therefore there is not a satisfactory explanation for the failure of DNA of these mutants to be packaged into capsids.

Mutants belonging to complementation group J assemble two types of DNA-containing particles, which band at a bouyant density of 1.36 and 1.34gms/ml, as well as the empty capsids, but fail to assemble particles with bouyant density of 1.26gms/ml. Analysis on alkaline sucrose gradients of particles with bouyant density 1.36 and 1.34gms/ml, revealed that the DNA present was of unit length. Examination of the polypeptide composition of these two types of particles by SDS-polyacrylamide gel electrophoresis showed that the heavier particles lacked penton base, protein V, pVI, pVII and pVIII, while the lighter particles lacked penton base and had reduced amounts of proteins V, pVI, pVII and pVIII, compared to WT virions. Since the fiber polypeptide comigrates with proteins pVI and pVIII it was impossible to determine whether it also was missing, but it is difficult to envisage how it could be present in the absence of penton base.

Immunoprecipitation of cell lysates revealed the absence of immunologically reactive penton bases in cells infected by group J mutants at 39.5°C, while the amount of fibers precipitated was comparable to that in WT lysates. Upon shift of cultures from 39.5°C to 32°C, all 4 mutants of this complementation group required synthesis of new proteins to form virions. However, all viral polypeptides, including those which make the multimeric penton base, made at 39.5°C were stable on prolonged chase at this temperature, suggesting that the penton base polypeptides made by group J mutants at the non-permissive temperature were incorrectly folded and were unable to re-fold upon shift a to the permissive temperature.

These data, together with the physical mapping localization of the lesions present in these mutants, strongly suggest them to be mutants of the penton base gene.

Further studies of the late IIIa protein mutants and characterization of the Ad7 penton and fiber polypeptides are described in the 1981-82 annual report.

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